

Ultraviolet-A (UVA-1) radiation suppresses immunoglobulin production of activated B lymphocytes *in vitro*

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Summary

Previous studies have shown that low-dose ultraviolet-A (UVA-1) total body irradiations were capable of improving disease activity in patients with systemic lupus erythematosus (SLE). We hypothesized that UVA-1-induced suppression of immunoglobulin production by activated B cells in the dermal capillaries could be (partly) responsible for this effect. Our experiments with donor skin demonstrated that approximately 40% of UVA-1 could penetrate through the epidermis. Irradiation of peripheral blood mononuclear cells (PBMCs) with 2 J/cm² of UVA-1 resulted in 20% cell death. This toxic effect could be prevented totally by preincubation of the cell cultures with catalase. This indicates that the generation of hydrogen peroxide plays a role in UVA-1 cytotoxicity. T cells and B cells appeared to be less susceptible to UVA-1 cytotoxicity than monocytes. With the use of a CD40–CD40L B cell activation method we measured immunoglobulin production after various doses of UVA-1 irradiation (0–2 J/cm²). The doses of 2 J/cm² caused a significant decrease of IgM, IgG, IgA and IgE production under the conditions of interleukin (IL)-10 or IL-4 (IgE) stimulation. Although UVA-1 can cause apoptosis of B lymphocytes, we show that relatively low doses of UVA-1 radiation also affect the function of these cells. Both effects may be responsible for the observed improvement of disease activity in SLE patients.

Keywords: B cells, immunoglobulins, skin, SLE, UV radiation

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Introduction

Systemic lupus erythematosus (SLE) is a relatively common, chronic disease characterized by the production of multiple antibodies. Although the pathogenesis of this multi-organ disease remains unclear, B lymphocytes are held largely responsible for the immune dysregulation that underlies the disease process [1]. A significant proportion of therapeutic strategies in SLE are based on decreased production or the selective removal of circulating autoantibodies [2,3].

About 10 years ago, long-wave (340–400 nm) ultraviolet (UVA) radiation, designated as UVA-1, was introduced as a potential therapeutic modality for SLE patients [4–7]. The development of this new approach in the treatment of SLE was contrary to the conventional knowledge of UV radiation being harmful to most patients with lupus erythematosus. The discovery by McGrath Jr and coworkers [8] that UVA radiation had a favourable effect on disease activity and

survival in a mouse model of SLE gave the first impetus to research in this new area. Later, promising results of uncontrolled and controlled studies of UVA-1 therapy in SLE patients were published by the same author [4–7]. Decreased disease activity scores, sometimes accompanied by lowered autoantibody titres, were reported. From this work it has also become clear that UVA-1, but not UVB or visible light, was responsible for the beneficial effects.

We have recently conducted two double-blind, placebo-controlled cross-over studies in 11 and 12 SLE patients, respectively, using so-called UVA-1 cold light irradiation equipment [9,10]. Being aware of the risk of photosensitivity in SLE patients, we applied a low dose (6 J/cm² in the first, 12 J/cm² in the second study) of UVA-1 radiation daily, 5 days a week for 3 weeks. Even though we used UVA-1 equipment different from the apparatus used by McGrath *et al.* we could confirm the beneficial effect of UVA-1 treatment on disease activity and the absence of side-effects in

both studies. In four patients with anti-Sjögren's syndrome-A (anti-SSA) antibodies decrease of titres was recorded after UVA-1 therapy in the first study [9]. In the second study the anti-SSA titre of one patient and the anti-ribonucleoprotein (anti-RNP) titre of another showed a marked decrease [10].

Whereas the same dose of short-wavelength UV light (UVB) would cause serious burns with many apoptotic cells in the superficial skin, UVA-1 in such a dose does not generate any macroscopic or microscopic changes in the epidermis or dermis. In the present work, we show that UVA-1 photons penetrate easily to the superficial dermis which enables them to affect the function of circulating lymphocytes, monocytes and other cells in the capillary network of the skin. In addition, we have found evidence that one of the mechanisms underlying the beneficial effect of UVA-1 in SLE patients could be a suppression of antibody production in activated B cells.

Materials and methods

Penetration of UVA-1 through the epidermis

Three pieces of normal Caucasian skin (skin types II–III) were received after cosmetic breast reduction. The skin was washed three times with phosphate-buffered saline (PBS) and subcutaneous fat was removed mechanically with small scissors. Each piece of skin was cut into three smaller parts (approximately 1.5×1.5 cm) which were incubated overnight with 3 ml dispase solution (Life Technologies BV, Breda, the Netherlands) in a Petri dish at 4°C. The next day, the contents of the Petri dishes were further incubated for 1 h at 37°C for 1 h, after which the dermis was separated from the epidermis with two small tweezers [11]. The epidermis was placed on a microscope cover glass (23×32 mm), washed with PBS to remove the rest of the dispase solution and kept in a Petri dish with a small amount of PBS to prevent desiccation.

The small pieces of epidermis were put onto cover glasses and placed on the aperture of a ultraviolet A-1 (UVA-1) measurement device (BioSun Sylt Service GmbH, Germany, <http://www.biosunsylt.com>). The epidermal sheets were large enough to cover the opening of the measurement device completely. By varying the distance between the lamps and the cell cultures, three different irradiances of UVA-1 (23, 31 and 47 mW/cm²) were applied and the percentage of penetrated UVA-1 radiation was determined. A BioSun Medical 500 000 UVA-1 cold-light unit (BioSun Sylt Service GmbH, Germany) was used as a UVA-1 source for these penetration experiments. The same unit was used for the irradiation of SLE patients in our previous study [10]. The irradiance measured behind an empty cover glass put on the device's aperture was considered as being 100% penetration. Each measurement was performed in triplicate.

Determination of UVA-1 toxicity on peripheral blood mononuclear cells (PBMCs) *in vitro*

The toxic effect of UVA-1 radiation was determined by evaluating the viability of irradiated PBMCs with the use of trypan blue exclusion (Sigma, USA). The cells of three healthy volunteers from the city of Leiden in the Netherlands were isolated from heparinized blood by Ficoll-Hypaque density-gradient ($\rho = 1.077$ g/ml; Pharmacia Biotech, Uppsala, Sweden) centrifugation and cultured in 24-well plates. The wells were exposed to 0.5–10 J/cm² of UVA-1 radiation. To one of every three wells catalase (Sigma), in a final concentration of 20 units/ml, was added prior to irradiation. Twenty-four hours after exposure to UVA-1 10 µl of trypan blue solution was added to each well and the cells were transferred to counting chambers to be counted manually. Counts were performed in triplicate.

To detect differences in the susceptibility to UVA-1 toxicity of the various cell populations of the PBMCs, the viability of UVA-1 irradiated CD3 positive (T cells), CD14 positive (monocytes) and CD20 positive cells (B cells) was determined. PBMCs were irradiated with 0, 0.5 and 2 J/cm² UVA-1. Twenty-four hours later, cell death in the different cell populations was identified by using propidium iodide and flow cytometric analysis [12].

Effect of UVA-1 on immunoglobulin production

PBMCs were obtained from heparinized blood of six healthy donors by separation on Ficoll-Hypaque ($\rho = 1.077$ g/ml, Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation.

PBMCs were cultured in T75 flasks (Greiner, Alphen aan den Rijn, the Netherlands), on a layer of γ -irradiated mouse fibroblasts transfected with human CD40L, or on non-transfected (control) mouse fibroblasts (L cells) [13]. They were maintained in Iscove's modified Dulbecco's medium with glutamax (IMDM; GIBCO BRL, Breda, the Netherlands), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; GIBCO BRL), 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Boehringer, Mannheim, Germany).

Recombinant human cytokine interleukin (IL)-4 (200 units/ml) or IL-10 (50 ng/ml, PeproTech, Rocky Hill, NJ, USA) was added to the cultures to evaluate the effect of these cytokines on immunoglobulin production.

Fifty thousand PBMCs were cultured on a layer of 5000 γ -irradiated (70 Gy) feeder cells: L-CD40L cells or L cells. The cultures were carried out in triplicate in 96-well culture plates at 37°C and 5% CO₂ saturation. The total volume (cytokines included) was 200 µl.

Half the tissue culture plates were irradiated daily with 0.5 or 2 J/cm² during the first week (i.e. five irradiations). After correction for the absorption of UVA-1 by the culture wells, these doses corresponded to exposure times of 12 and 48 s, respectively. The other half of the culture plates received the

same doses of UVA-1 during the second week of incubation. All supernatants were collected on day 15. IgM, IgG, and IgA production resulting from all conditions was measured by enzyme-linked immunosorbent assay (ELISA) [14], IgE production was determined by radioimmunoassay (RIA) [15]. A paired *t*-test was used to evaluate differences between immunoglobulin production after 0, 0.5 and 2 J/cm² UVA-1 irradiation. Statistical significance was defined as *P* = 0.05.

The experiments were repeated with and without catalase added to the culture wells 30 min prior to UVA-1 irradiation. These cultures were irradiated in the second week of incubation. IgM, IgG, and IgA production resulting from these conditions was measured. Again, a paired *t*-test was used to evaluate differences between immunoglobulin production after 0, 0.5 and 2 J/cm² UVA-1 irradiation, and to assess differences between catalase and noncatalase conditions.

Results

Penetration of UVA-1 through the epidermis

In order to obtain an estimate of the proportion of UVA-1 radiation that can reach the superficial dermis where blood capillaries are present, we investigated the penetration of UVA-1 through the epidermis. Despite increasing irradiances, the penetration of UVA-1 through the epidermis of donor skin remained constant for all skin pieces (Fig. 1). However, interindividual variations in UVA-1 penetration ranged from 25% to 50%. The average penetration calculated from a total of 27 measurements was $39 \pm 12\%$, which implies that approximately 60% of UVA-1 radiation was absorbed by the epidermis.

Determination of UVA-1 toxicity on PBMCs *in vitro*

After we had determined that a considerable part of UVA-1 irradiation was indeed capable of reaching the dermal layers

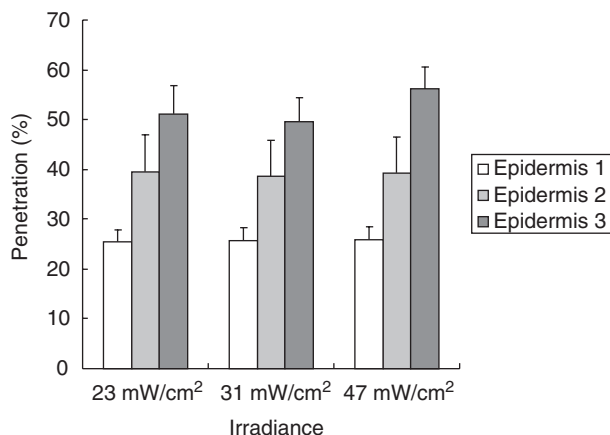


Fig. 1. Mean penetration of three different irradiances of ultraviolet (UVA)-1 (23, 31 and 47 mW/cm²) through three different pieces of epidermis from normal Caucasian people (skin types II–III). The columns show means \pm s.d.

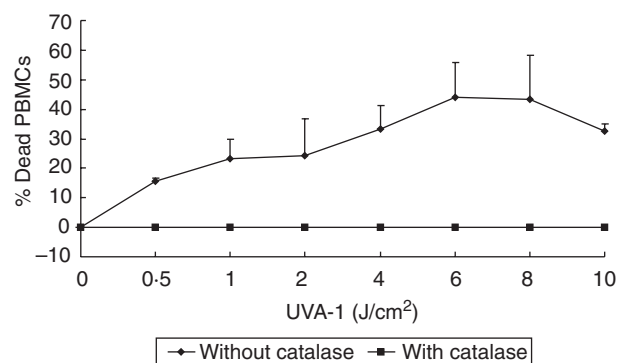


Fig. 2. The cytotoxic effect of ultraviolet (UVA)-1 on peripheral blood mononuclear cells (PBMCs), expressed as the mean percentage of dead PBMCs determined by trypan blue exclusion after a single irradiation with 0.5–10 J/cm² of UVA-1 radiation, in the presence and absence of catalase (20 units/ml). The values are shown as means \pm s.d.

of the skin and its capillaries, we started studying the cytotoxic effect of UVA-1 on PBMCs *in vitro*. As determined by trypan blue exclusion, increasing UVA-1 doses resulted in an increasing portion of non-viable PBMCs (Fig. 2). Preincubation of the cells with catalase totally prevented the toxic effects of UVA-1 radiation, suggesting the involvement of hydrogen peroxide in UVA-1 toxicity. The shape of the toxicity curve of UVA-1 (Fig. 2) created the impression of a stepwise increase of cytotoxicity. This might be explained by the selective death of different PBMC subpopulations with increasing doses of UVA-1. Flow cytometric analysis was used to find out whether there were differences in the sensitivity of different PBMC subpopulations to low doses of UVA-1 radiation. The proportion of viable CD20 positive cells (B cells) was constant at 0.5 and 2 J/cm² and slightly increased at 10 J/cm² (Fig. 3a,b), whereas the proportion of CD3 positive cells (T cells) increased slightly. As the proportion of viable CD14 positive cells decreased with increasing UVA-1 dose, monocytes seemed to be the most sensitive cells.

Effect of UVA-1 on immunoglobulin production

Because the B cell population appears to remain relatively invariable after low doses of UVA-1 *in vitro*, we investigated whether these UVA-1 irradiations result in decreased immunoglobulin production by activated B cells *in vitro*. In order to examine the effect of UVA-1 radiation on immunoglobulin production in peripheral blood B cells, we used the well-established CD40L culture system [13]. PBMCs were cultured on a layer of γ -irradiated mouse fibroblasts transfected with human CD40L in the absence or presence of recombinant cytokines IL-4 or IL-10. In previous studies, the group of Banchereau *et al.* showed that IL-4 is essential for IgE production and that IL-10 is a critical factor for B cell activation and subsequent IgM, IgG and IgA (but not IgE) production [16,17]. In this culture system, B cell activation consists of a

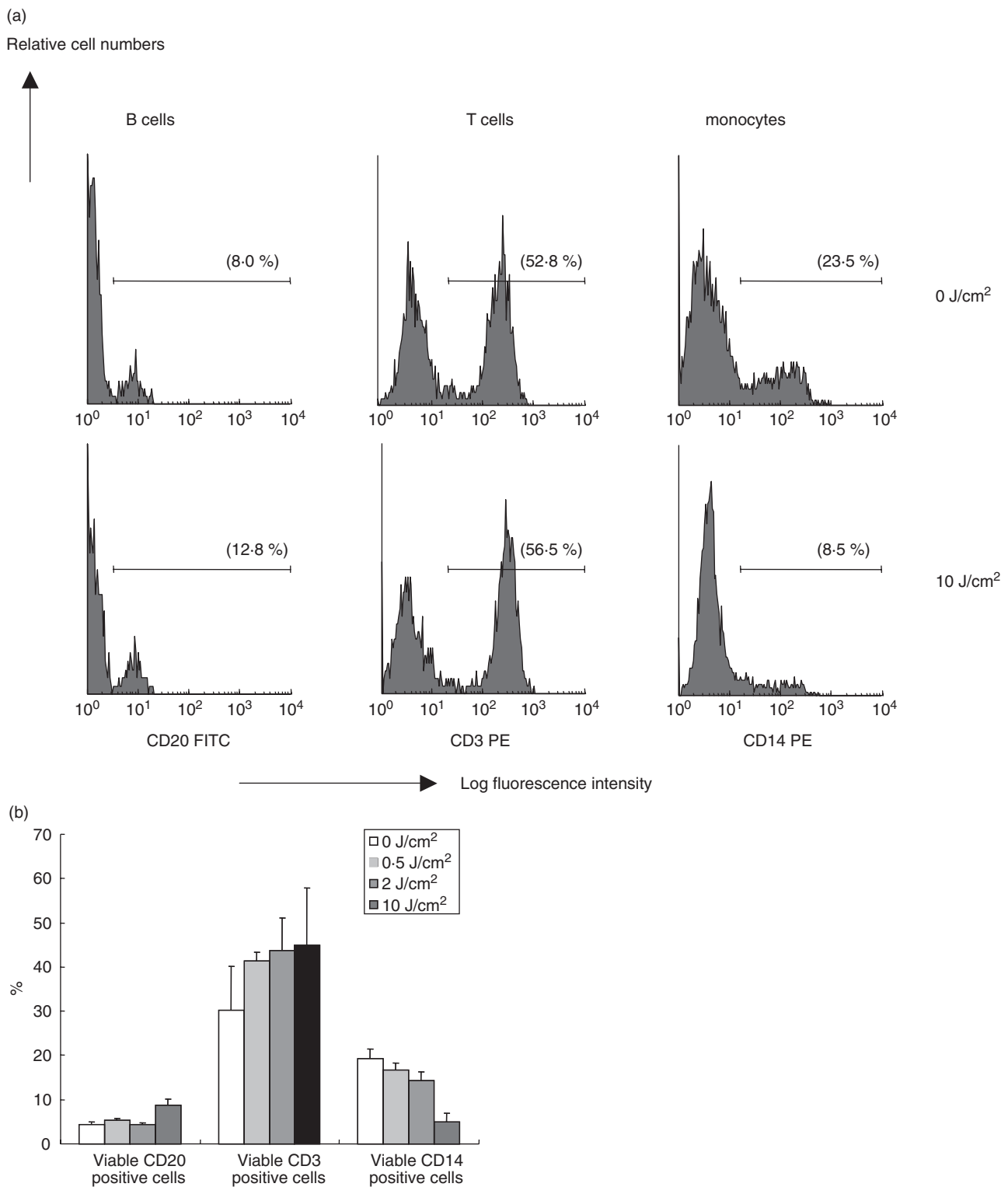


Fig. 3. (a) Fluorescence activated cell sorter (FACS) analysis detecting B lymphocytes (CD20), T lymphocytes (CD3) and monocytes (CD14) of peripheral blood mononuclear cells (PBMCs) before and after the ultraviolet (UVA)-1 irradiation (10 J/cm²) [as used for the calculation of the data shown in (b)]. (b) The proportion of viable CD3 positive (T lymphocytes), CD14 positive (monocytes) and CD20 positive cells (B lymphocytes) 24 h after irradiation with 0, 0.5, 2 and 10 J/cm² UVA-1, determined by flow cytometric analysis. The values are presented as means \pm s.d.

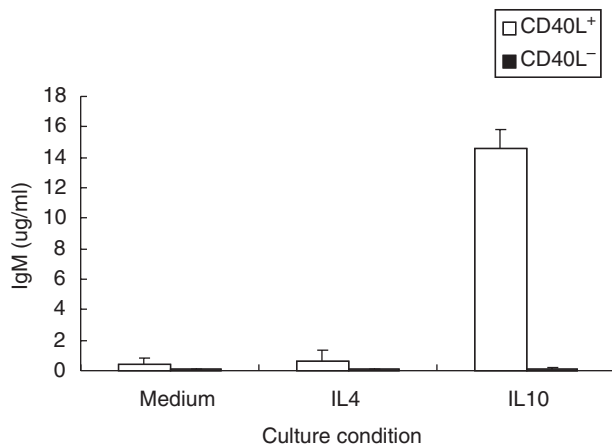


Fig. 4. IgM production in non-irradiated cultures, after 2 weeks' incubation of CD40L positive fibroblasts and fibroblasts lacking CD40L, under interleukin (IL)-4- or IL-10-stimulated or non-stimulated culture conditions. Data are shown as means \pm s.d.

first period of proliferation (week 1) followed by a second period of differentiation and antibody production [13]. Immunoglobulin production at the end of the first week of incubation was generally very low (not shown). In cultures of fibroblasts lacking CD40-L and in those without added cytokines immunoglobulin production at the end of the second week was also very low (see Fig. 4 for IgM). The combination of CD40L with IL-10 resulted in significant production of IgM at this time-point. At day 15, IgE production was present in the IL-4 stimulated conditions, suggesting that isotype switching took place during the second week of incubation (not shown).

Daily UVA-1 irradiation in the first week did not affect immunoglobulin production in the supernatants at day 15. However, UVA-1 exposure of the cultured cells during the second week of incubation resulted in a dose-dependent decrease of IgM, IgG and IgA production in IL-10 stimulated conditions and IgE in IL-4 stimulated conditions (Fig. 5).

To investigate whether the decrease of immunoglobulin production after UVA-1 can be prevented by catalase, we repeated some of these experiments in the presence and absence of catalase. A statistically significant dose-dependent decrease of immunoglobulin production was observed for all isotypes tested, confirming the results described above. However, there were no significant differences in immunoglobulin production between the conditions with and without catalase (Fig. 6).

Discussion

Our experiments demonstrate that approximately 40% of UVA-1 reaches the dermis where it may influence various components, including the circulating cells in the capillaries. UVA-radiation, even in a relatively low dose, appears to be harmful for some white cells. Our investigations show that a

dose of 2 J/cm² UVA-1 caused around 20% death of PBMCs. This toxic effect increased further with rising UVA-1 doses. However, preincubation with catalase totally prevented this UVA-1-induced cell death, suggesting that generated hydrogen peroxide plays an important role in this UVA-1-induced toxicity.

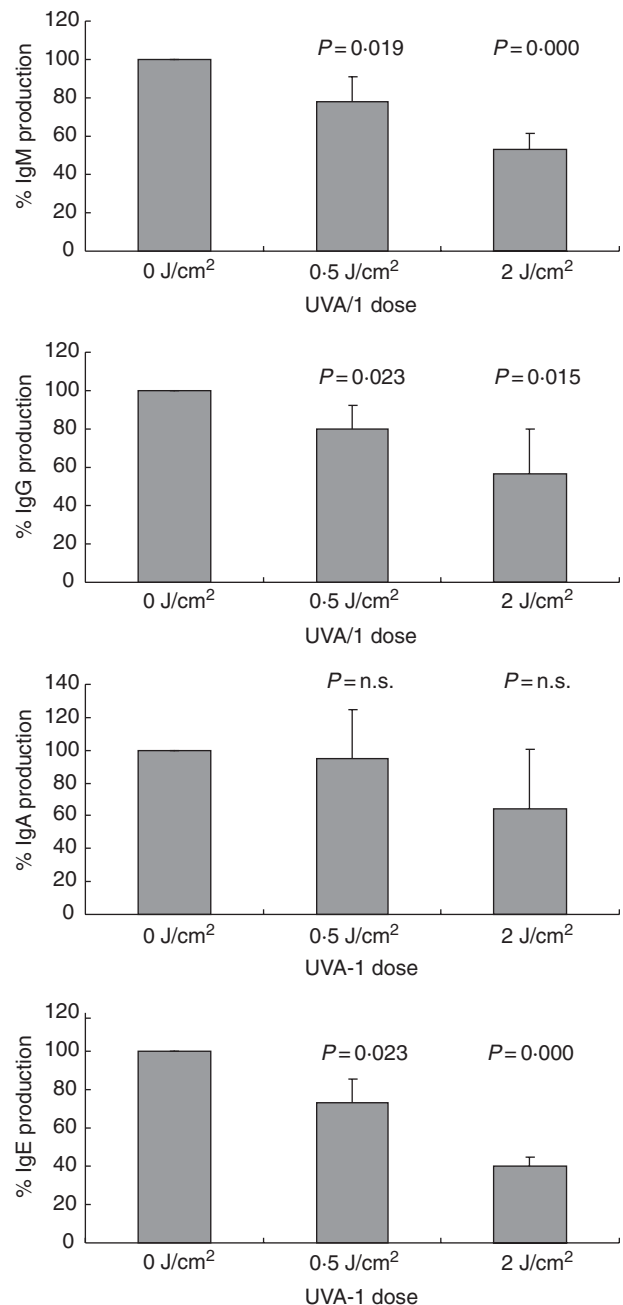


Fig. 5. The inhibitory effect of 0.5 or 2 J/cm² ultraviolet (UVA)-1 on IgM, IgG, IgA and IgE production in supernatants of peripheral blood mononuclear cells (PBMCs) cultures activated with CD40L and interleukin (IL)-10, during the second week of incubation. Data are expressed as means \pm s.d. of the changes in the immunoglobulin production expressed in percentages.

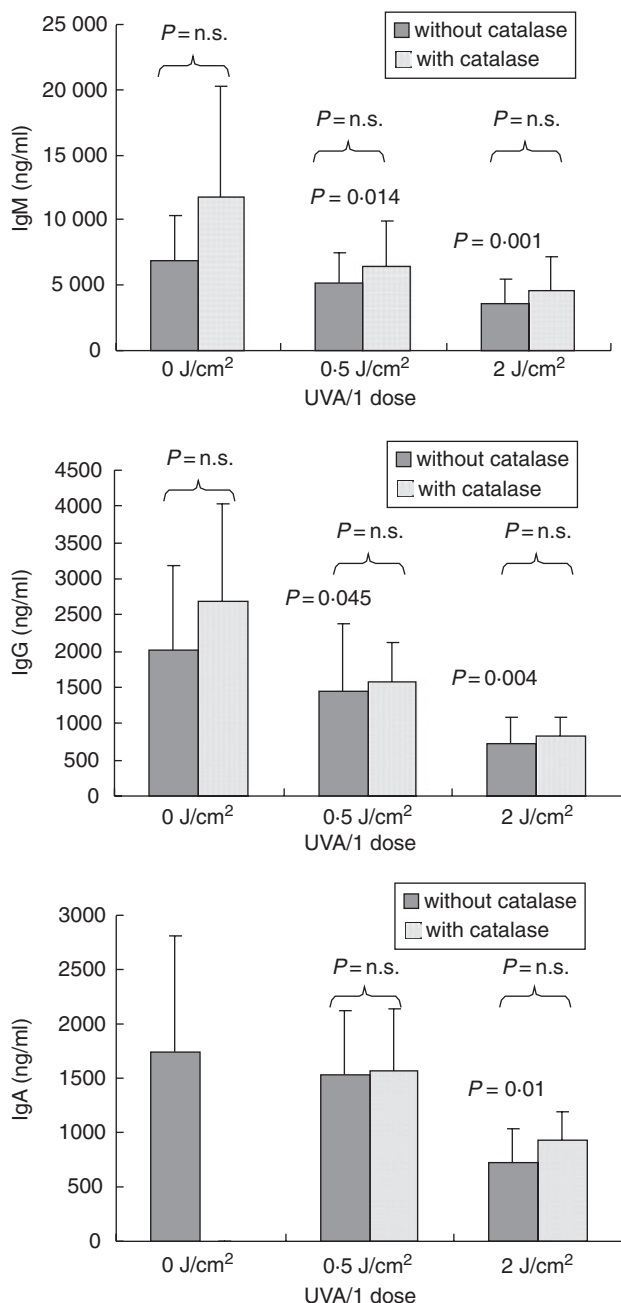


Fig. 6. The effect of added catalase on IgM, IgG and IgA production in supernatants of peripheral blood mononuclear cell (PBMC) cultures activated with CD40L and interleukin (IL)-10 after 2 J/cm² ultraviolet (UVA)-1 during the second week of incubation.

Absorption of UVA-1 by its chromophores (such as porphyrines or riboflavins) can lead to photosensitization reactions that result in production of reactive oxygen species, singlet oxygen and superoxide radicals. The latter can undergo dismutation to hydrogen peroxide [18]. As the highest concentration of the mentioned UVA-1 absorbing compounds is present in mitochondria, these organelles are likely to be the most UVA-1 sensitive cellular targets. Mitochondrial injury leads to decreased ATP production, which

in turn influences many synthetic processes. Many ATP molecules are necessary for protein synthesis. One can expect that even minor oxidative damage of mitochondria in activated B cells could consequently lead to decreased protein (immunoglobulin) production. Reactive oxygen species can also lead to apoptosis of B cells through activation of the caspase pathway by cytochrome c. Singlet oxygen is able to open mitochondrial megachannels, releasing apoptosis initiating factor (AIF) and cytochrome c [19].

According to Farber *et al.* [20], B cells are more sensitive to hydrogen peroxide than T cells. In our fluorescence activated cell sorter (FACS) experiments the sensitivity of three PBMC types was as follows: CD14 > CD20 and CD3 (Fig. 3). The B cell population consists of 60% naive cells and 40% CD27-positive memory B cells [21]. Recently, Jacobi *et al.* [22] showed that the number of circulating CD27^{high} plasma cells correlated with disease activity in SLE patients. It would be interesting to investigate whether there is a difference between the cytotoxic effect of UVA-1 on different B cell populations.

A dose-dependent decrease of immunoglobulin production was observed after UVA-1 radiation during the second week in the IL-10 or IL-4 stimulated conditions. IgE concentrations in the supernatants were substantially lower than IgM concentrations (not shown). This can be explained by the fact that CD40–CD40L binding with IL-4 stimulation results in B cell proliferation and IgE isotype switching, whereas CD40–CD40L binding with IL-10 stimulation gives rise not only to B cell proliferation and IgG and IgA isotype switching, but also to plasma cell differentiation with increased immunoglobulin production. Plasma cell differentiation primarily takes place in the second week of cell culture, which explains the fact that only very low immunoglobulin concentrations could be measured in both non-irradiated and irradiated conditions at day 8 (not shown).

Twenty per cent of cell death in the PBMC population was observed 24 h after exposure to 2 J/cm² UVA-1. However, immunoglobulin production following daily irradiations with the same dose of UVA-1 in the second week was more than 20% reduced. An impaired B cell function could be responsible for this difference, or the cumulative effect of daily irradiations resulting in more cell death could be the cause. In the latter situation, the favourable effect *in vivo* could be longer-lasting.

In additional experiments the effect of catalase on immunoglobulin production was investigated. Again, a significant dose-dependent decrease of immunoglobulin production was observed. However, no significant effect of catalase could be discerned. This observation could possibly be explained by the fact that catalase removes hydrogen peroxide exclusively extracellularly. This enables it to prevent UVA-1 induced cell death by lipid peroxidation of the outer cell membrane, as hydrogen peroxide, in contrast with catalase, can penetrate the cell membrane. However, extracellular

catalase apparently does not have any profound effect on the intracellular concentration of UVA-1 induced hydrogen peroxide.

Because the epidermis absorbs a considerable portion of UVA-1 irradiation, doses higher than 2 J/cm² are probably needed to reach a therapeutic effect. In our clinical studies, we utilized 6 and 12 J/cm². According to our penetration experiments, these doses would correspond to approximately 2.4 and 4.8 J/cm² of UVA-1 reaching the dermal capillaries. Therefore, the effects of the doses used in our *in vitro* experiments were relevant to the situation in our previous clinical trials.

In conclusion, we have found evidence that long-wave UVA radiation, after penetration of the epidermis, is able to lower the production of antibodies in activated B cells and plasma cells. This effect can (partly) explain the clinical improvement observed in SLE patients after UVA-1 therapy.

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